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A multiplex PCR assay for the identification of animal species in feedstuffs

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Abstract

A multiplex Polymerase Chain Reaction (PCR) assay was applied to feedstuff analysis for the identification of the most used species in rendering plants (ruminant, poultry, fish and pork materials). Primers were designed in different regions of mitochondrial DNA (12S rRNA, tRNA Val and 16S rRNA) after alignment of the available sequences in the GenBank database. The primers generated specific fragments of 104–106, 183, 220–230 and 290 bp length for ruminants, poultry, fish and pork, respectively. The detection limit was 0.004% for fish primers and 0.002% for ruminants, poultry and pork primers.

The multiplex PCR proposed in this study can be considered a valid alternative to the microscopic method for the detection of animal derived materials banned by a European Union Regulation as a preventive measure against the spread of Bovine Spongiform Encephalopathy.

Keywords: Feedstuff; Meat and bone meal; Species identification; Multiplex polymerase chain reaction

1. Introduction

A ban of animal derived meals in the manufacturing of feedstuffs has been introduced by a European Community Regulation [1] as a preventive measure to avoid the spread of Bovine Spongiform Encephalopathy (BSE).

The detection of animal tissues in feedstuffs has therefore gained great interest in the last few years.

For this purpose, a microscopic method, based on the analysis of animal bone fragments, has been developed. This method has been recognized as the 'official' method in the European strategy against the BSE. However, it is time consuming, requires specialised staff and only enables the detection of zoological classes (mammalian, avian and fish), while the species origin of bone fragments remains undetermined.

The need for alternative analytical approaches has prompted numerous studies. Biomolecular techniques have been extensively investigated as they offer undoubtful advantages, such as having a high degree of specificity and being applicable even to heat processed products [2]. Although DNA like proteins undergoes thermal denaturation, it has been observed that DNA can be still detected by short fragment amplification [3].

Polymerase Chain Reaction (PCR) has been applied for the detection of bovine tissue in animal feedstuffs [4–7].

Lahiff et al. [8] developed a PCR to recognize ovine, porcine and poultry DNA in feedstuffs.

Myers et al. [9] identified different species in feedstuffs using universal primers coupled with restriction endonucleases.

More recently, Bottero et al. [10] developed a method which involved the ability of primers to amplify wider target sequences. This PCR based assay demonstrated to be highly sensitive and useful in routine feedstuff analysis for the detection of all vertebrates.

This method could be further improved with a one step PCR detection and discrimination of the most used species in rendering plants.

The aim of the present study was therefore to develop a multiplex PCR for the rapid identification of ruminant, poultry, fish and pork materials.

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2. Materials and methods

2.1. Samples

Samples of raw meat and autoclave treated meat (121 °C for 15 min) from different species of earthly and aquatic origin were analyzed. Each sample weighed 3 g. The species considered are listed in Table 1.

Four commercial meals, four pet food and five baby food were also analyzed. The commercial meals were obtained from a rendering plant, while pet food and baby food were obtained from the retail trade (Table 1).

In order to evaluate the test sensitivity, a DNA mix of the four species (bovine, chicken, European pilchard and pork) was diluted in vegetable DNA (maize) up to 0.001%.

Finally, known amounts of a bovine blood meal, prepared in our laboratory under controlled conditions, were diluted (1, 0.5, 0.1%) in a vegetable meal (maize and soy) and accurately mixed. DNA was extracted from each diluted samples.

Table 1
Samples submitted to the assay

Samples	Species
Ruminant meat	*Bos taurus*
	Capra hircus
	Ovis aries
Avian meat	*Gallus gallus*
	Meleagris meleagridis
Fish meat	*Oncorhynchus mykiss*
	Sardina pilchardus
	Engraulis encrasicolus
	Mullus sp.
	Sebastes sp.
	Lophius piscatorius
	Scomber scombrus
	Boops boops
	Trigla sp.
	Merlangius merlangus
	Acipenser sp.
	Solea solea
Pork meat	*Sus scrofa*
(A) Blood meal	Bovine
(B) Meat meal	Bovine
(C) Meat meal	Pork
(D) Meat meal	Fish
(A) Pet food	Fish
(B) Pet food	Chicken/turkey
(C) Pet food	Chicken/turkey
(D) Pet food	Bovine/turkey
(A) Baby food	Bovine
(B) Baby food	Chicken
(C) Baby food	Turkey
(D) Baby food	Fish
(E) Baby food	Pork

2.2. DNA extraction

Dneasy Tissue kit (Qiagen, Hilden, Germany), with minor modifications for the application to complex products, was used for extraction of DNA from all samples. The modifications consisted of an increase of the sample amount from 25 to 400 mg and a decrease of the final elution volume, reduced to 100 μl.

The DNA was quantified by spectrophotometry (Biophotometer 6131, Eppendorf AG, Hamburg, Germany).

2.3. Primers design

Species primers were designed from different regions of mitochondrial DNA (12S rRNA, tRNA Val and 16S rRNA) following alignment of available sequences from GenBank database with version 1.6 of Clustal W [11] (Table 2).

The primers were synthesized by Roche Diagnostic, Monza, Italy.

2.4. Simplex PCR

In a preliminary phase of this investigation, primers specificity was assessed with DNA extracted from the species panel listed in Table 1.

PCR amplification was performed in a final volume of 50 μl containing 75 mM Tris–HCl (pH 8.8), 1 unit of Platinum Taq DNA Polymerase (Invitrogen, USA), 0.1 mg/ml BSA (Roche Diagnostics GmbH, Mannhein, Germany), 0.2 mM each of dATP, dCTP, dGTP, dTTP (Pharmacia, Uppsala, Sweden), 2 mM $MgCl_2$, 25 pmol of primers and 250 ng of DNA template.

Amplification was performed in a Thermal Cycler 2400 (Applied Biosystems, Foster City, CA) with the following cycling conditions; after an initial heat denaturation step at 94 °C for 10 min, 35 cycles were programmed as follows: 94 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min and final extension at 72 °C for 5 min.

2.5. Multiplex PCR

For the simultaneous detection of each species, a one step multiplex PCR was developed using each of the primer sets previously designed for the simplex PCR.

As for the simplex PCR, amplification was performed in a final volume of 25 μl containing 75 mM Tris–HCl (pH 8.8), 1.5 unit of Platinum Taq DNA Polymerase (Invitrogen, USA), 0.1 mg/ml BSA (Roche Diagnostics GmbH, Mannhein, Germany), 0.2 mM each of dATP, dCTP, dGTP, dTTP (Pharmacia, Uppsala, Sweden), 2 mM $MgCl_2$, 20, 20, 12.5 and 10 pmol of ruminant, pork, fish and poultry primers, respectively, and 125–625 ng of DNA template. The choice of template concentration depended on the nature of the sample: in the case of feedstuffs, where a low concentration of animal DNA is expected, 625 ng were

Table 2
Design of oligonucleotides of the different animal species

Primers	Species[a]	Genes	Positions	Oligonucleotides primers	Amplicons (bp)
Ruminant	*Bos taurus*	16S rRNA	*Bos taurus*	5′ GAA AGG ACA AGA GAA ATA AGG 3′	104
	Capra hircus		[b]NC 001567	5′ TAG GCC CTT TTC TAG GGC A 3′	
	Ovis aries		2920		
			3023		
Pork	*Sus scrofa*	12S rRNA-tRNA Val	*Sus scrofa*	5′ CTA CAT AAG AAT ATC CAC CAC A 3′	290
			[b]NC 000845	5′ ACA TTG TGG GAT CTT CTA GGT 3′	
			1971		
			2260		
Fish	*Sardinops melanostictus*	12S rRNA	*Sardinops melanostictus*	5′ TAA GAG GGC CGG TAA AAC TC 3′	224
	Sardinella hualiensis		[b]NC 002616	5′ GTG GGG TAT CTA ATC CCA G 3′	
	Pagrus major		291		
	Tracurus japonicus		514		
Poultry	*Gallus gallus*		*Gallus gallus*	5′ TGA GAA CTA CGA GCA CAA AC 3′	183
	Meleagris meleagridis	12S rRNA	[b]NC 001323	5′ GGG CTA TTG AGC TCA CTG TT 3′	
			1799		
			1981		

[a] Species considered for sequences alignment.
[b] Accession number Genbank.

used. Thermal cycling was programmed following the same procedure used in simplex PCR.

Amplimers were resolved by electrophoresis on 3% agarose gel (Invitrogen, USA) run in Tris Acetate EDTA Buffer for 70 min at 110 V and stained with Ethidium Bromide (0.4 ng/ml) for 20 min.

2.6. Sequencing

Multiplex PCR fragments of the expected length were purified by Qiaquick Gel Extraction Kit (Qiagen, Hilden, Germany) and cycle sequenced (both strands) using PCR derived primers and the dideoxy chain termination method, with fluorescence dye terminators (Applied Biosystem, Foster City CA). Sequenced fragments were resolved by capillary electrophoresis using an ABI 310 Genetic Analyser (Applied Biosystems, Foster City CA). The sequences obtained were deposited in the Genbank database under accession number AY309496-99.

The nucleotide sequences were submitted to BLASTn [12] sequence similarity searching at the National Centre for Biotechnology Information database and were aligned with the bovine, pilchard, pork and chicken sequences available in GenBank database.

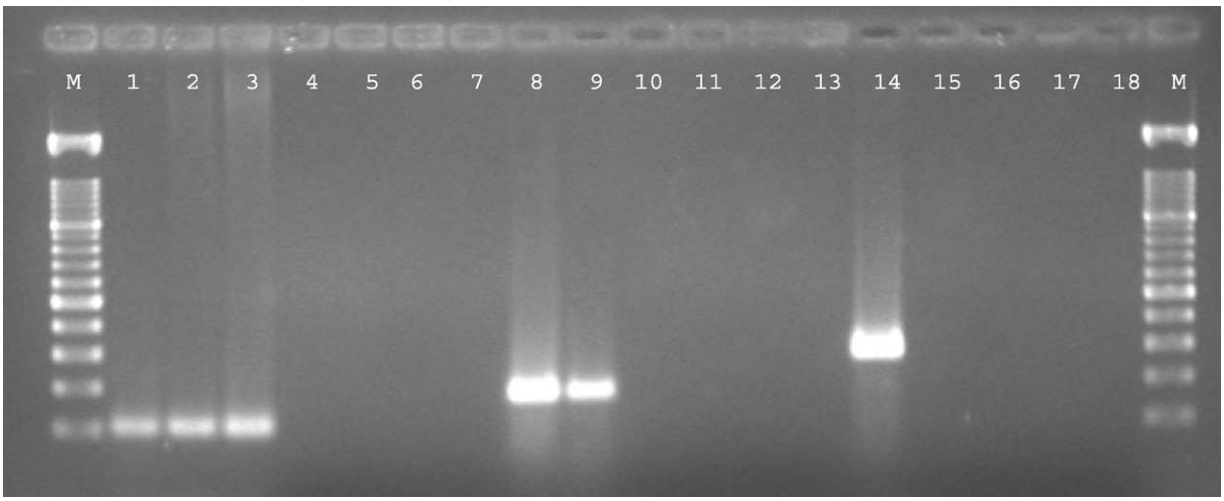

Fig. 1. Specificity of simplex PCR of DNA from raw meat with ruminant primers (lanes 1–7), poultry primers (lanes 8–13) and pork primers (lanes 14–18). Lane 1, *Bos taurus*; lane 2, *Capra hircus*; lane 3, *Ovis aries*; lane 4, *Gallus gallus*; lane 5, *Oncorhynchus mykiss*; lane 6, *Sus scrofa*; lane 7, control reagent; lane 8, *Gallus gallus*; lane 9, *Meleagris meleagridis*; lane 10, *Bos taurus*; lane 11, *Oncorhynchus mykiss*; lane 12, *Sus scrofa*; lane 13, control reagent; lane 14, *Sus scrofa*; lane 15, *Bos taurus*; lane 16, *Gallus gallus*; lane 17, *Oncorhynchus mykiss*; lane 18, control reagent; M, 100-bp ladder.

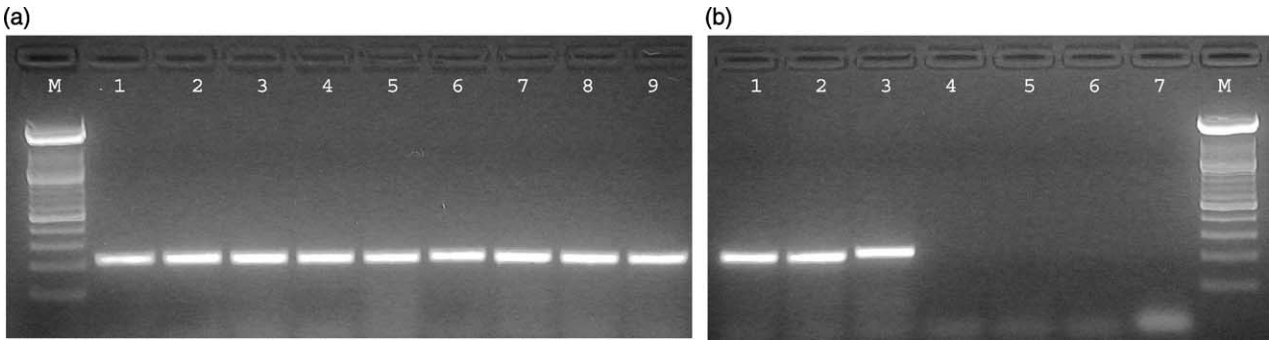

Fig. 2. (a) Specificity of simplex PCR of DNA from raw meat with fish primers. Lane 1, *Sardina pilchardus*; lane 2, *Oncorhynchus mykiss*; lane 3, *Trigla* sp.; lane 4, *Sebastes* sp.; lane 5, *Lophius piscatorius*; lane 6, *Engraulis encrasicolus*; lane 7, *Scomber scombrus*; lane 8, *Boops boops*; lane 9, *Merlangius merlangus*; M, 100-bp ladder. (b) Specificity of simplex PCR of DNA from raw meat with fish primers. Lane 1, *Mullus* sp.; lane 2, *Solea solea*; lane 3, *Acipenser* sp.; lane 4, *Bos taurus*; lane 5, *Gallus gallus*; lane 6, *Sus scrofa*; lane 7, control reagent; M, 100-bp ladder.

3. Results

3.1. Simplex PCR specificity

In a preliminary phase of the investigation, simplex PCRs were carried out on DNA extracted from raw meat to verify the specificity of the primers. The primers generated specific fragments of 104–106 bp for ruminants, 183 bp for avians, 220–230 bp for fishes and 290 bp for pork. The size of ruminant and fish fragments depended on the number of deletions in each species.

To detect possible cross-reactions, each set of primers was challenged in simplex PCR with non-target species. In no case a cross-reaction was observed (Figs. 1 and 2a,b).

In addition, to verify test reproducibility on degraded DNA, autoclave treated meat samples were tested.

Every test, repeated three or more times, gave reproducible results.

3.2. Multiplex PCR specificity

When multiplex PCR was carried out on analogous samples, the set of primers retained the same specificity, the result of which may be seen in Fig. 3. The electrophoretic pattern clearly shows the absence of cross-reaction. In fact, only the species specific band is evident.

3.3. Sequencing

The results of sequencing are summarized in Table 3. The similarity score obtained from ovine, chicken and pork sequences generated in our study revealed 100% homology with *Ovis aries*, *Gallus gallus* and *Sus scrofa* sequences available in GenBank, respectively. On the contrary, the European pilchard sequence obtained in this assay showed a low similarity (87%) because the reference GenBank species was *Sardinops melanostictus*, while *Sardina pilchardus* was analyzed.

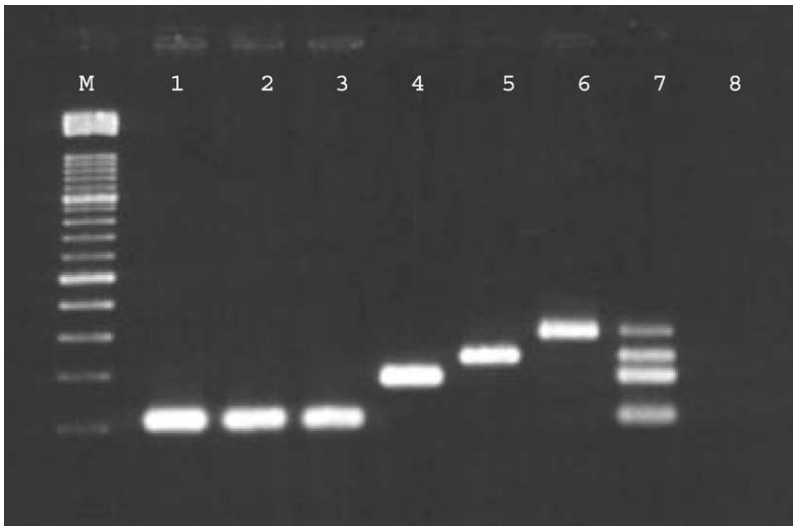

Fig. 3. Specificity of multiplex PCR of DNA from raw meat of: lane 1, *Bos taurus*; lane 2, *Capra hircus*; lane 3, *Ovis aries*; lane 4, *Gallus gallus*; lane 5, *Oncorhynchus mykiss*; lane 6, *Sus scrofa*; lane 7, mix of *Bos taurus, Gallus gallus, Oncorhynchus mykiss* and *Sus scrofa* DNA; lane 8, control reagent; M, 100-bp ladder.

Table 3
Sequencing results of multiplex PCR products

Sequences deposited	GenBank sequences	Homology rate (BLASTn) (%)
Ovis aries AY 309496	AF 010406	100
Gallus gallus AY 309497	AY 235571	100
Sardina philcardus AY 309498	AB 032554[a]	87
Sus scrofa AY 309499	AF 304203	100

[a] *Sardinops melanostictus*.

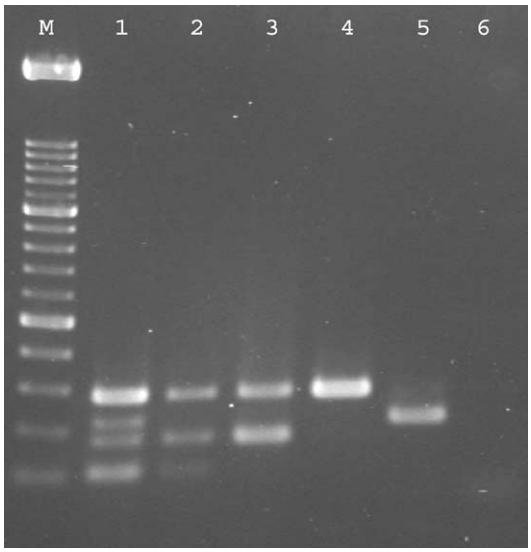

Fig. 4. Multiplex PCR on commercial meals. Lane 1, positive control; lane 2, bovine meat meal (B); lane 3, bovine blood meal (A); lane 4, pork meat meal (C); lane 5, fish meat meal (D); lane 6, control reagent; M, 100-bp ladder.

3.4. Applicability of multiplex PCR

The applicability of the assays to commercial meals, pet food and baby food has been demonstrated. The results may be seen in Figs. 4 and 5 and in Table 4 which reports the true species composition of the listed sample products.

With regard to commercial meals, only in fish meal the species claimed in label has been confirmed by the DNA analysis.

As for pet food, the claimed species has always been detected, apart from the case of pet food A in which in addition to fish, the presence of poultry has been evidentiated. In pet food C the indication 'animal fat' can be related to the presence of pork DNA.

Finally, in all the tested baby food only the species claimed in label was detected.

3.5. PCR sensitivity

PCR amplifications was performed on mixed template DNA, dilutions of which were performed in maize. The detection limit was 0.004% for fish primers and 0.002% for ruminants, poultry and pork primers (Fig. 6).

Furthermore, by means of different dilutions of known amounts of a bovine blood meal in a vegetable meal (maize and soy) it was possible to reveal a sensitivity threshold of 0.1% (Fig. 7).

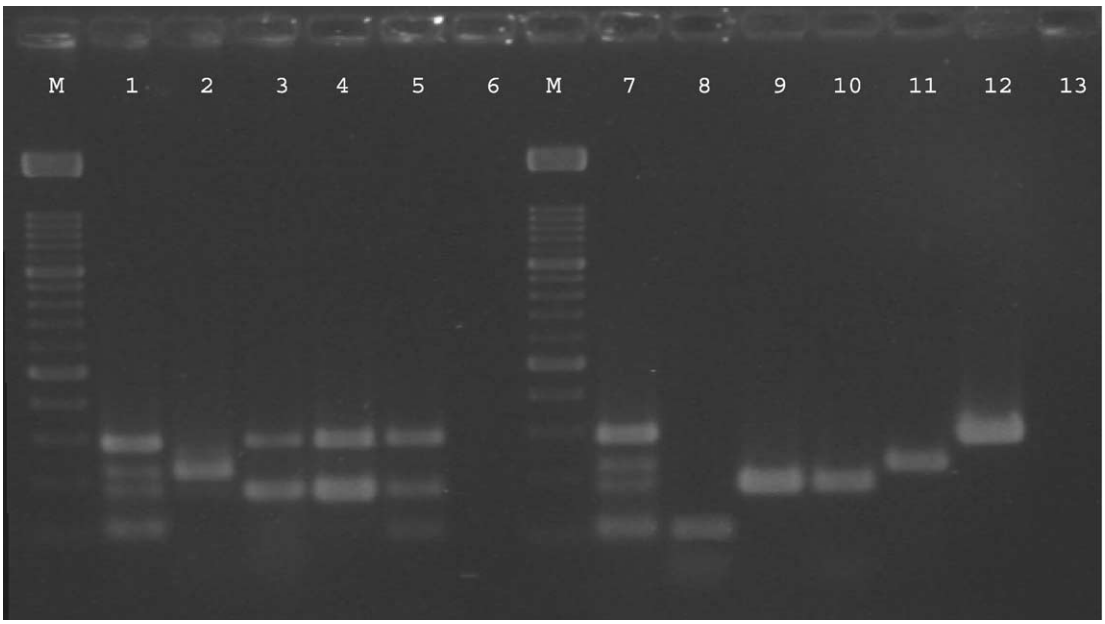

Fig. 5. Multiplex PCR on pet food and baby food. Lane 1, positive control; lane 2, pet food (A); lane 3, pet food (B); lane 4, pet food (C); lane 5, pet food (D); lane 6, control reagent; lane 7, positive control; lane 8, baby food (A); lane 9, baby food (B); lane 10, baby food (C); lane 11, baby food (D); lane 12, baby food (E); lane 13, control reagent; M, 100-bp ladder.

Table 4
Results of PCR performed on commercial meals, pet food and baby food

Product	Label	Result
(A) Blood meal	Bovine	Pork/poultry
(B) Meat meal	Bovine	Pork/poultry/ruminant
(C) Meat meal	Pork	Pork/poultry
(D) Meat meal	Fish	Fish
(A) Pet food	Fish	Fish/poultry
(B) Pet food	Various meats including: chicken meat minimum 4%, turkey meat minimum 4%	Poultry/pork
(C) Pet food	Chicken/turkey/animal fat	Poultry/pork
(D) Pet food	Various meats including: bovine meat minimum 4%, turkey meat minimum 4%	Ruminant/poultry/pork
(A) Baby food	Bovine	Bovine
(B) Baby food	Chicken	Poultry
(C) Baby food	Turkey	Poultry
(D) Baby food	Fish	Fish
(E) Baby food	Pork	Pork

4. Discussion

At present, a critical point concerning the observance of EU ban of animal meals in feedstuffs is represented by the reliability of the control tests. The low resolution efficiency of the microscopic method, which allows the detection of zoological classes but not of species, highlights the need for alternative analytical approaches.

Technologies based on DNA analysis seem to fulfil this need.

The present paper describes the development and application of a multiplex PCR to detect ruminant, poultry, fish and pork materials in feedstuffs in a single reaction step.

Primers were designed addressing different genes of mitochondrial DNA characterized by alternate well-conserved regions and much variable regions. Primer binding sites (PBS) were selected in order to generate specific amplimers of less than 300 bp in length. In this way it is possible to apply the assay to samples whose DNA is highly degraded by heat following the treatment indicated by the EU Law for thermal processing of animal ingredients (134.4–141.9 °C and 3.03–4.03 bar for 24 min). This degradation has caused some drawbacks in PCR application as observed by Bottero et al. [10].

Ruminant primers were designed in a well-conserved region for bovine, caprine and ovine species for the detection of ruminant material prohibited in the manufacturing of feedstuffs [1].

The poultry primer set was designed for the specific detection of poultry species (chicken, turkey).

Fish primers, designed following the alignment of four teleosteans sequences available in GenBank (Table 2), amplified wide range of teleosteans species as expected (Fig. 2a,b). However, to confirm the specificity of amplicons obtained, we aligned our primers with the sequences of *Oncorhynchus mykiss, Engraulis encrasicolus, Lophius piscatorius, Scomber scombrus, Acipenser* sp. and *Solea solea*. A high degree of homology (98–100%, depending on the species) in PBS was observed. Further, the distance between the two sense and antisense PBS was nearly 220 bp, which is consistent with the fish amplicon length.

The multiplex PCR described in this paper proved to be very sensitive, with a very low detection limit when DNA mixtures were tested. The same assay, applied on experimental mixtures of bovine blood meals in vegetable, showed the same detection limit of the microscopic official method [13]. The possibility of detecting lower levels of animal DNA in feedstuffs would be interesting from a theoretical point of view, but not helpful in practice. In fact,

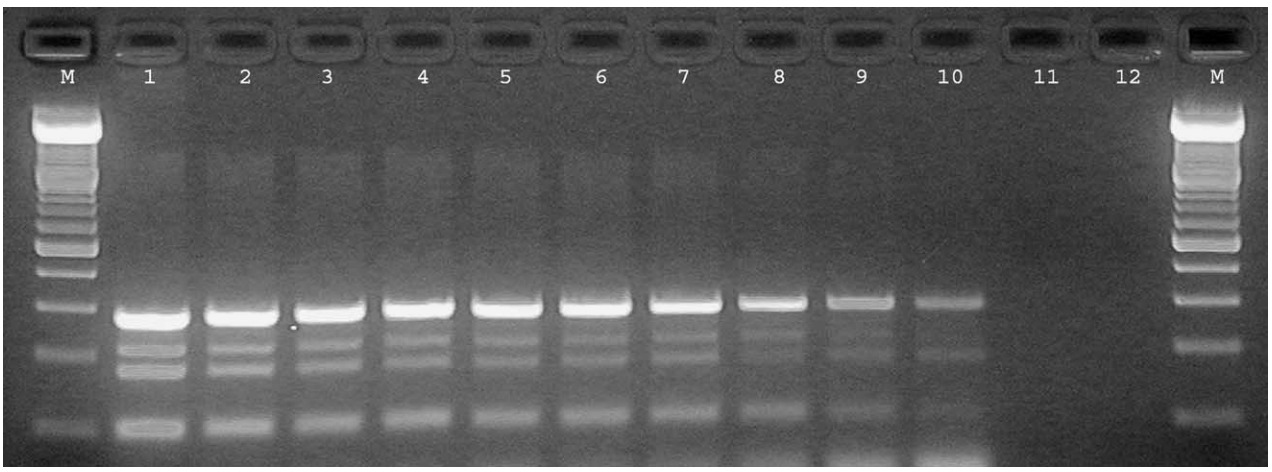

Fig. 6. Evaluation of assay sensitivity: progressive dilution of a mixed DNA template diluted in DNA of maize. Lane 1, 100%; lane 2, 10%; lane 3, 1%; lane 4, 0.2%; lane 5, 0.1; lane 6, 0.066%; lane 7, 0.033%; lane 8, 0.0167%; lane 9, 0.004%; lane 10, 0.002%; lane 11, 0.001%; lane 12, control reagent; M, 100-bp ladder.

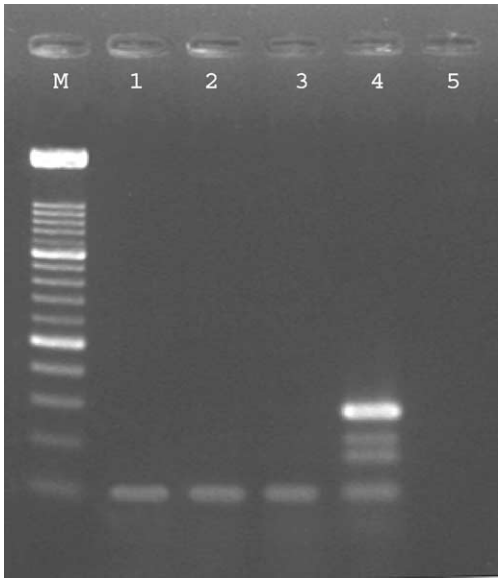

Fig. 7. Evaluation of assay sensitivity: progressive dilution of know amounts of a bovine blood meal in a vegetable meal (maize and soy). Lane 1, 1%; lane 2, 0.5%; lane 3, 0.1%; lane 4, positive control; lane 5, control reagent; M, 100-bp ladder.

in case of very small amounts of contaminating animal materials, it could be difficult to establish whether a fraud is presumable or an unintentional contamination occurred. This drawback could be overcome by means of a quantitative PCR. Lahiff et al. [14] developed a real-time PCR able to quantify the presence of bovine material in feedstuff samples.

As for samples shown in Table 4, the results of PCR performed on commercial meals in use before the 'BSE ban', put in evidence the presence of animals species not indicated on the label. Clearly, at the moment of the manufacturing of these products less rigorous laws in this field did exist.

This result has suggested an extension of the assay to other items from the retail trade, such as pet food and baby food.

The data of Table 4 demonstrate a careful choice of raw materials in the manufacturing of baby food, while in pet food the choice is not so accurate.

In conclusion, the multiplex PCR proposed in this study can be considered as a further improvement of a PCR based assay for the control of feedstuffs. It can be applied to obtain more detailed informations when a preliminary test with universal primers has given positive results.

The test could be useful in the control of different products, such as baby food, to verify the origin of the raw materials, especially in products submitted to denaturing technologies, for which other methods cannot be applied.

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